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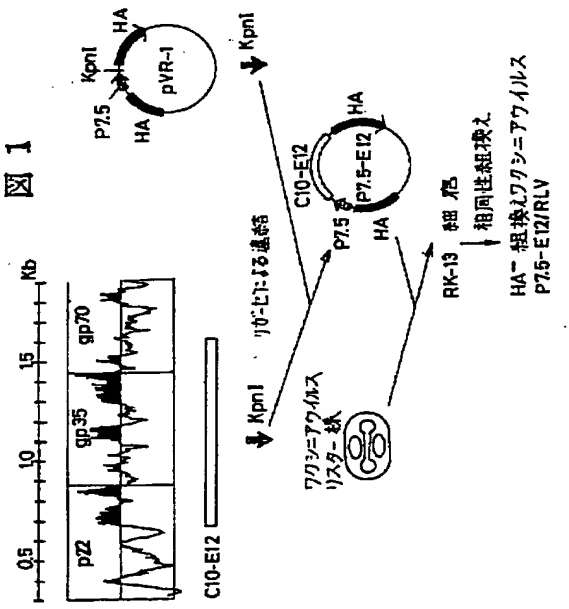
(54)【発明の名称】　組換えワクシニアウイルスを用いる非Ａ非Ｂ型肝炎ウイルス遺伝子の発現および非Ａ非Ｂ型肝炎ワクチン

(57)【要約】　(修正有)

【目的】本発明は、組換えワクシニアウイルスを用いる非Ａ非Ｂ型肝炎ウイルス抗原蛋白質の発現およびワクチンへの応用。

【構成】非Ａ非Ｂ型肝炎ウイルス抗原蛋白質をコードする塩基配列を含む遺伝子と、該遺伝子を発現させ得るウイルスプロモーターと、ワクシニアウイルスの増殖のために必須でないワクシニアウイルス遺伝子とを含有するプラスミド、該プラスミドとワクシニアウイルスの相同性組換えにより得られた組換えワクシニアウイルス及びその製造方法、該組換えワクシニアウイルスからの発現非Ａ非Ｂ型肝炎ウイルス抗原蛋白質の製造方法及び糖を有するその発現産物、並びに、糖を有する発現非Ａ非Ｂ型肝炎ウイルス抗原蛋白質又は弱毒化若しくは不活化組換えワクシニアウイルスを含むワクチン。

図1



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【特許請求の範囲】

【請求項1】　非A非B型肝炎ウイルス抗原蛋白質をコードする塩基配列を含む遺伝子と、該遺伝子を発現させ得るウイルスプロモーターと、及びワクシニアウイルスの増殖のために必須でないワクシニアウイルス遺伝子とを含有するプラスミド。

【請求項2】　前記抗原蛋白質がウイルス構造蛋白質である請求項1記載のプラスミド。

【請求項3】　前記構造蛋白質がエンベロープ糖蛋白質である請求項2記載のプラスミド。

【請求項4】　前記ウイルスプロモーターがワクシニアウイルス由来のプロモーターである請求項1記載のプラスミド。

【請求項5】　前記ワクシニアウイルス遺伝子がヘマグルチニン遺伝子である請求項1記載のプラスミド。

【請求項6】　前記抗原蛋白質をコードする塩基配列を含む遺伝子及び前記ウイルスプロモーターの1組以上が、前記ワクシニアウイルス遺伝子中に挿入されていることを特徴とする請求項1記載のプラスミド。

【請求項7】　プラスミドP7．5－E12。

【請求項8】　請求項1～7のいずれか一項に記載のプラスミドとワクシニアウイルスの相同性組換えにより得られた組換えワクシニアウイルス。

【請求項9】　組換えワクシニアウイルスP7．5－E12／RLV。

【請求項10】　請求項8又は9記載の組換えワクシニアウイルスの製造方法であって、

請求項1～7のいずれか一項に記載のプラスミドを制限的に線状化し、ワクシニアウイルスが感染している動物細胞にトランスフェクションして相同性組換えを行う段階、及び　非A非B型肝炎ウイルス抗原蛋白質をコードする塩基配列を含む遺伝子が挿入されている組換えウイルスをスクリーニングし、回収する段階を含む方法。

【請求項11】　ワクシニアウイルスがワクシニアウイルス・リスター株である請求項10記載の方法。

【請求項12】　前記動物細胞がウサギ腎継代細胞RK－13である請求項10記載の方法。

【請求項13】　請求項8又は9記載の組換えワクシニアウイルスを動物細胞に感染し、該細胞を培養して非A非B型肝炎ウイルス抗原蛋白質をコードする塩基配列を含む遺伝子を発現させ、発現蛋白質を回収することから成る発現非A非B型肝炎ウイルス抗原蛋白質の製造方法。

【請求項14】　前記動物細胞がウサギ腎継代細胞RK－13である請求項13記載の方法。

【請求項15】　請求項13又は14記載の方法によって得られる、糖を有する発現非A非B型肝炎ウイルス抗原蛋白質。

【請求項16】　請求項15記載の糖を有する発現非A非B型肝炎ウイルス抗原蛋白質を含む、非A非B型肝炎

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を治療又は予防するためのワクチン。

【請求項17】　弱毒化した請求項8又は9記載の組換えワクシニアウイルスを含む、非A非B型肝炎を治療又は予防するためのワクチン。

【請求項18】　不活化した請求項8又は9記載の組換えワクシニアウイルスを含む、非A非B型肝炎を治療又は予防するためのワクチン。

【発明の詳細な説明】

【0001】

【産業上の利用分野】本発明は、組換えワクシニアウイルスを用いる非A非B型肝炎ウイルス抗原蛋白質の発現およびワクチンへの応用に関する。

【0002】

【従来の技術】非A非B型肝炎はウイルス性の伝染性肝炎であり、発症後多くは慢性化し、肝硬変、肝癌へと高率に移行することから社会的にも大きな問題であり、その予防法が必要とされている。ウイルス性疾患の予防にはワクチンを用いる方法と感染状況を把握してその道すじを断つことの2種類がある。このうちで感染状況を把握するのに必要な診断用のキットはカイロン社のM. Houghtonら（特表平2－500880号公報）あるいは槇ら（特願平2－180889号）によりすでに開発されており、輸血による感染はかなり高率で予防できるようになりつつある。しかし非A非B型肝炎患者の約半数（約50万人／年）は輸血を介さない感染者であり、現在のところこれらの感染を予防する有効な手段はなく、予防ワクチンの開発が希求されている。

【0003】この種のワクチンに関してはすでに、M. Houghtonらが、上記公報中でヒトC型肝炎ウイルス（HCV）エピトープを含む免疫原性ポリペプチドを含有するワクチン、不活性化HCVを含有するワクチン、及び弱毒化HCVを含有するワクチンについて開示している。

【0004】

【発明が解決しようとする課題】通常、ワクチンにはホルマリン等の薬剤で不活化されたウイルスそのものが使われることが多いが、非A非B型肝炎ウイルスはいまだに分離されておらず、またin vitroでのウイルス増殖系もないため、大量に該ウイルスを得ることができない。そのため、非A非B型肝炎ウイルス遺伝子を大腸菌、酵母、動物細胞等で発現させ、ワクチン抗原を得ることが示唆されている（特表平2－500880号公報）。

【0005】一般に、ウイルスエンベロープ蛋白質のように糖をもつ蛋白質は、それをもたない蛋白質と比べて免疫原性が高く免疫化に優れているが、このような糖蛋白質の有効な発現法及びその取得については、M. Houghtonらは何ら教示していない。ウイルスエンベロープ蛋白質のような糖蛋白質を糖鎖が付加した形で発現させるのには動物細胞を用いた発現系が考えられる。しかし動物細胞を用いた発現系では一般的に数mg／lの効率でし

か発現蛋白質が得られず、ワクチンとして使用可能な精製度にまで純度をあげるのはかなり困難である。

【０００６】本発明者らは、鋭意研究の結果、ワクシニアウイルス遺伝子中に非Ａ非Ｂ型肝炎ウイルス抗原蛋白質をコードする塩基配列を含む遺伝子及びウイルスプロモーターを含有する組換えプラスミドとワクシニアウイルスとの間での相同性組換えにより得られる組換えワクシニアウイルスを用いることによって、糖をもつ非Ａ非Ｂ型肝炎ウイルス抗原蛋白質が発現され得ることを見出し、この発明を完成した。

【０００７】本発明は、組換えワクシニアウイルスを用いる非Ａ非Ｂ型肝炎ウイルス抗原蛋白質の発現系を提供することを目的とする。

【０００８】本発明はまた、この発現系を介して得られる天然型又は天然型に近い該抗原蛋白質を提供することを目的とする。

【０００９】本発明はさらに、弱毒化若しくは不活化組換えワクシニアウイルスおよび該抗原蛋白質のワクチンへの応用を目的とする。

【００１０】組換えワクシニアウイルスをワクチンとして使用する場合は、ウイルスが接種部位で増殖することにより免疫を与えるので非常に少量の使用でよく、精製も簡単である。

【００１１】

【課題を解決するための手段】本発明は、非Ａ非Ｂ型肝炎ウイルス抗原蛋白質をコードする塩基配列を含む遺伝子、該遺伝子を発現させ得るウイルスプロモーター、及びワクシニアウイルスの増殖のために必須でないワクシニアウイルス遺伝子を含有するプラスミドを提供する。

【００１２】該プラスミドは、ワクシニアウイルスとの間での相同性組換えにより組換えワクシニアウイルスを作製するうえで必須な構成物である。

【００１３】非Ａ非Ｂ型肝炎ウイルス抗原蛋白質をコードする塩基配列を含む遺伝子において、コードされる該抗原蛋白質は非Ａ非Ｂ型肝炎ウイルスの非構造蛋白質又は構造蛋白質のいずれでもよいが、効率的な免疫化を実現するためには構造蛋白質、特にエンベロープ及びコアを構成する蛋白質が好ましい。エンベロープは、動物ウイルス外被を構成する構造体であり、細胞質膜に類似した脂質二重層膜とウイルス遺伝子によって作られた糖蛋白質構造体とを含む。この糖蛋白質構造体は、該膜中にその一端が埋め込まれ、他端が突起状（スパイクと呼ばれる）に外部に突出している。また、コアは、内部カプシドとも呼ばれ、ウイルスゲノムを包む蛋白質から成る殻を構成している。該抗原蛋白質は糖を必ずしも必要としないが、免疫原性を高めるためには糖蛋白質であることが好ましく、この場合、糖蛋白質の効率的発現を可能にする本発明の特徴が十分に発揮され得る。後述の実施例では、実際、非Ａ非Ｂ型肝炎ウイルスのエンベロープ抗原糖蛋白質ｇｐ３５をコードする遺伝子をプラスミド

に組み込み、その発現を実現している。

【００１４】ウイルスプロモーターは、前記抗原蛋白質をコードする塩基配列を含む遺伝子を発現させ得るものであればその種類を問わない。このようなプロモーターとしては、ワクシニアウイルス由来プロモーター（７．５ｋＤ蛋白質、ＡＴＩプロモーター）、サイトメガロウイルスプロモーターなどが挙げられるが、組換えワクシニアウイルス内での該遺伝子の発現を有効に行わしめるためには、ワクシニアウイルス由来プロモーターが好ましい。

【００１５】ワクシニアウイルス遺伝子は、このウイルスの増殖系に係る遺伝子以外のものが好ましく、組換えウイルスのスクリーニングの点で特にヘマグルチニン（ＨＡ）遺伝子が好適に使用され得る。

【００１６】また、これらの遺伝子及びプロモーターの配置は、一般に、非Ａ非Ｂ型肝炎ウイルス抗原蛋白質をコードする塩基配列を含む遺伝子がウイルスプロモーターの下流に配置されるように、両者がワクシニアウイルス遺伝子内部に挿入された形態をとるのが適している。

【００１７】本発明の実施態様によるプラスミドＰ７．５－Ｅ１２が本発明プラスミドとして好適に使用し得、この特定プラスミドも本発明の範囲内に含まれる。この例示のプラスミドは、後述の実施例に示すように、先ずワクシニアウイルスＷＲ株よりＨＡ遺伝子を単離してｐＵＣ18にクローニングし、このＨＡ遺伝子のＮｒｕＩ部位にワクシニアウイルス由来の７．５ｋＤ蛋白質プロモーターを挿入し、その下流に非Ａ非Ｂ型肝炎ウイルス抗原蛋白質をコードする塩基配列を含む遺伝子を挿入することにより作製することができる（図１参照）。

【００１８】本発明はまた、上述の組換えプラスミドとワクシニアウイルスの相同性組換えにより得られた組換えワクシニアウイルスを提供する。

【００１９】本発明組換えワクシニアウイルスは、本発明の上記組換えプラスミドを制限的に線状化し、ワクシニアウイルスが感染している動物細胞にトランスフェクションして相同性組換えを行う段階、及び非Ａ非Ｂ型肝炎ウイルス抗原蛋白質をコードする遺伝子が挿入されている組換えウイルスをスクリーニングし、それを回収する段階を含む方法により製造することができる。この製造方法も本発明の範囲内に含まれる。

【００２０】本発明の実施態様により、ワクシニアウイルスとしてワクシニアウイルスリスター株が好適に使用され得る。また、該ウイルスを感染させるための動物細胞としては、哺乳動物細胞が適しており、例えばウサギ腎継代細胞（ＲＫ－１３細胞）を挙げることができる。

【００２１】本発明の実施態様による組換えワクシニアウイルスＰ７．５－Ｅ１２／ＲＬＶが好適に使用し得、これも本発明の範囲内に含まれる。この組換え体ウイルスにおいては、線状化されたプラスミドｐ７．５－Ｅ１２／ＲＬＶがワクシニアウイルス遺伝子中のＨＡ（ヘマ

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グルチニン）蛋白質をコードする遺伝子のほぼ中央に挿入されている。このため正常なヘマグルチニン蛋白質は産生されない。また挿入された非Ａ非Ｂ型肝炎ウイルス遺伝子は頭の部分に開始コドンＡＴＧをもつので、ここから新たにｍＲＮＡが合成され蛋白合成が行なわれる。

【００２２】組換えワクシニアウイルスのスクリーニングは、先ず赤血球吸着試験（ＨＡ試験）（Shida, H., virology 150:451-462, 1986 ）によりＨＡ（－）の組換えウイルス候補株を取得し、次いで非Ａ非Ｂ型肝炎ウイルス抗原蛋白質を構成するペプチドをコードするヌクレオチド配列をプローブとするプラークハイブリダイゼーションによって陽性クローンを検出する手法により実施し得る。

【００２３】本発明はさらに、上述のようにして作製された組換えワクシニアウイルスを動物細胞に感染し、該細胞を培養して非Ａ非Ｂ型肝炎ウイルス抗原蛋白質をコードする塩基配列を含む遺伝子を発現させ、発現蛋白質を回収することから成る発現非Ａ非Ｂ型肝炎ウイルス抗原蛋白質の製造方法を提供する。

【００２４】動物細胞は哺乳動物細胞が適しており、特にウサギ腎継代細胞（ＲＫ－１３細胞）が好適に使用され得る。また、培養条件は使用する動物細胞に依存して決定され、増殖可能な培地、培養温度、培養時間等が適宜選択される。例えば前記Ｐ７．５－Ｅ１２／ＲＬＶの場合、グルコースの代りにフルクトースを含む牛血清含有ＥＭＥＭ培地が使用され得る。

【００２５】非Ａ非Ｂ型肝炎ウイルス抗原蛋白質の発現は、該抗原蛋白質に対する抗体か又はそれを含有する非Ａ非Ｂ型肝炎患者血清を用いる免疫沈降法及び間接蛍光抗体法のような慣用の免疫学的技術によって確認され得る（図２および図３）。

【００２６】本発明はまた、このようにして発現された非Ａ非Ｂ型肝炎ウイルス抗原蛋白質のうち、特に糖を有する発現非Ａ非Ｂ型肝炎ウイルス抗原蛋白質を提供する。

【００２７】糖蛋白質の発現は、非Ａ非Ｂ型肝炎ウイルス関連分野においてはいまだに実現されたことがなく、該抗原糖蛋白質をコードする塩基配列を含む遺伝子を含有する組換えワクシニアウイルスを使用する発現系の発明によってはじめて該遺伝子の発現を可能にした。

【００２８】本明細書中、『糖を有する発現非Ａ非Ｂ型肝炎ウイルス抗原蛋白質」とは、天然型非Ａ非Ｂ型肝炎ウイルス抗原糖蛋白質およびその類似体の両方を包含することを意味する。また、該天然型抗原糖蛋白質の類似体は、非Ａ非Ｂ型肝炎ウイルスの動物体内への侵入を阻止することが又は、動物に感染した該ウイルスを撲滅することが可能な非Ａ非Ｂ型肝炎ウイルスに対する抗体を産生し得る範囲の改変体を意味する。

【００２９】本発明はさらに、上記の糖を有する発現非Ａ非Ｂ型肝炎ウイルス抗原蛋白質を含む、非Ａ非Ｂ型肝

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炎を治療又は予防するためのワクチンを提供する。

【００３０】本発明はまた、弱毒化した又は不活化した組換えワクシニアウイルスを含む、非Ａ非Ｂ型肝炎を治療又は予防するためのワクチンを提供する。

【００３１】ワクシニアウイルスはヒトの痘瘡免疫をつくるのに使われるポックスウイルス属の一種である。組換えワクシニアウイルスを動物の細胞、組織、器官等の中で増殖させた後、病原性を弱めて弱毒化生ワクチンとしたり、又はホルムアルデヒドで不活化して不活化ワクチンとすることができる。後述の実施例７では、組換えワクシニアウイルスＰ７．５－Ｅ１２／ＲＬＶをウサギ背部皮内に接種することにより、実際、ウサギ血清中に非Ａ非Ｂ型肝炎ウイルス抗原蛋白質に対する抗体が産生されることが実証される（図４）。

【００３２】本発明のワクチンは、一般に、免疫原が油中水乳剤中に含まれるか又は水酸化アルミニウム、リン酸アルミニウム等の無機ゲル上に吸着されるか又は慣用の有機アジュバントを含む等のアジュバントワクチンの形態かあるいは生理食塩水、グリセロール等の液体溶媒中の溶液形態で使用されるが、これに限定されない。また、ワクチンの適用法としては接種が好ましく、目的とする予防効果又は治療効果が得られる量で投与され、その量は個体の年齢、体重、症状等に依存する。

【００３３】

【実施例】以下の実施例により、本発明を更に詳細に説明するが、本発明は本発明の要旨を変えない限りこれらの実施例に限定されるものではない。

【００３４】実施例　１

ワクシニアウイルスのヘマグルチニン（ＨＡ）遺伝子のクローニング

ワクシニアウイルスＷＲ株をショ糖密度勾配遠心法［Joklik.W.K,Virology, 18, 9-18 (1962) ］で精製し、50mM Tris-HCl(pH 7.4) バッファー（1mM EDTA、0.5%ドデシル硫酸ナトリウム含有）中に懸濁し、プロティナーゼＫを 250～1000μｇ／mlの濃度になるように加えて３７℃で一晩インキュベートした後、ＴＥバッファーで飽和下フェノール：クロロホルム（１：１）液で３回抽出し、エタノール沈殿を行ないウイルスＤＮＡを得た。このＤＮＡを中塩濃度緩衝液中でHindIII により消化し、アガロースゲル電気泳動により、約５０ｋｂのHind IIIA断片を得た。このHind IIIA断片を、高塩濃度緩衝液中でＳａｌＩにより消化し、Hind IIIA断片の３′末端に位置する約１．８ kbpのHind III－ＳａｌＩ断片を、アガロースゲル電気泳動により単離した。

【００３５】一方、プラスミドｐＵＣ１３を中塩濃度緩衝液中でHindIII により、続いて高塩濃度緩衝液中でＳａｌＩにより消化して線状化した。この線状化プラスミドをアガロースゲル電気泳動により、単離した。

【００３６】前記のHind III－ＳａｌＩ断片と、線状化プラスミドとをライゲーション緩衝液中でＴ４リガーゼ

により連結し、この反応混合物を用いて大腸菌ＪＭ103を形質転換した。個々の形質転換体からアルカリ抽出法により、プラスミドを回収した。さらに制限酵素による分析を行ない、目的とする遺伝子構成を有するプラスミドを選択し、これをｐＨＡ１３と命名した。

【００３７】実施例　２

ワクシニアウイルスの７．５ｋＤタンパク質プロモーターを用いた組み換え用マルチベクターの作製

ワクシニアウイルスの７．５ｋＤタンパク質プロモーターとその下流に連結した外来遺伝子をワクシニアウイルスのゲノムＨＡ遺伝子内に組み込むための組み換え用ベクターを以下の方法で作製した。

【００３８】まず、ワクシニアウイルスWR株よりＤＮＡを実施例１で示した方法と同様に抽出し、Venkatesanらの方法［Venkatesan & B. Moss, J. Virol. 33, 738-745(1981)］により７．５ｋＤタンパク質プロモーターを単離した。すなわち、抽出したWR株のＤＮＡをＳａｌＩにて消化し、得られた０．９ｋｂｐのＤＮＡ断片をｐＵＣ１８のＳａｌＩサイトにクローニングした。さらに、このプラスミドをＲｓａＩおよびＨｉｎｃＩＩで消化し、７．５ｋＤタンパク質プロモーターを含む0.26 kbpのＲｓａＩ－ＨｉｎｃＩＩ断片を得た。このＤＮＡ断片をｐＵＣ１８にクローニングした（ｐ 7.5−18）。

【００３９】次に、以上の方法により単離した７．５ｋＤタンパク質プロモーターを実施例１でクローニングしたＨＡ遺伝子のＮｒｕＩサイトに以下の方法で挿入した。

【００４０】プラスミドｐ 7.5−18を中塩濃度緩衝液中でＥｃｏＲＩおよびＨｉｎｄＩＩＩ により消化することにより、約0.29 kbpの 7.5ｋプロモーター遺伝子を切り出し、アガロースゲル電気泳動により単離した。単離した遺伝子断片をニックトランスレーション緩衝液中でｄＴＴＰ、ｄＣＴＰ、ｄＡＴＰおよびｄＧＴＰ存在下においてKlenow断片と反応させ末端を平滑にした。

【００４１】一方、プラスミドｐＨＡ−13をＮｒｕＩ緩衝液中で消化して、線状化した。この線状化したプラスミドと前記の平滑化した遺伝子断片をライゲーション緩衝液中でＴ４リガーゼで連結し、大腸菌ＪＭ109 を形質転換した。形質転換体から得られたプラスミドは、制限酵素による分析の結果、７．５ｋＤプロモーター（P7.5）がＨＡ遺伝子の方向と同じ向きに挿入されたものであった。これをｐＶＲ－１と命名した（図１）。

【００４２】実施例　３

組換えプラスミドの作製

非Ａ非Ｂ型肝炎ウイルスのエンベロープ領域の遺伝子をワクシニアウイルスのゲノムのＨＡ遺伝子中に組み込むために用いる組換えベクターを以下の方法で作製した。

【００４３】非Ａ非Ｂ型肝炎ウイルスの構造蛋白質をコードする領域をもつクローンＣ10－Ｅ12（微工研条寄第3444号）（特願平２－413844号）を低塩濃度緩衝液中でＫｐｎＩにて消化し、約1.0kbpの遺伝子断片をアガロースゲル電気泳動により単離し遺伝子断片を得た（図１）。

【００４４】また、組換え用プラスミドｐＶＲ－１を低塩濃度緩衝液中で同じくＫｐｎＩにて消化し、線状化した。両者をライゲーション緩衝液中でＴ４リガーゼにより連結した。この連結物を用いて大腸菌ＨＢ101 を形質転換し、形質転換体よりアルカリ法にてプラスミドを回収した。制限酵素による分析を行ない、エンベロープ領域がプロモーターの下流に同方向に連結されているプラスミドを得、これをＰ７．５－Ｅ１２と命名した。

【００４５】実施例　４

組換えワクシニアウイルスの作製

ａ）トランスフェクションに用いるＤＮＡの調製

DIAGENプラスミドキット（DIAGEN社製）を用いて、２００mlの培養液で培養した大腸菌から１５０μｇのプラスミドP7.5 －E12を抽出した。このプラスミドをＣｓＣｌ密度勾配遠心法により精製した。すなわち、プラスミドをρ＝1.47ｇ／mlのＣｓＣｌ液（ＥｔＢｒ含有）に溶解し、クイックシールチューブ（ベックマン社製、ウルトラクリアー、１３×５１mm）に充填したものを、10℃、55,000rpm で１５時間遠心した。（VTi 65.2ロータ－、ベックマン超遠心機）。遠心後、closed circular プラスミドＤＮＡのバンドを回収し、イソプロパノール抽出を３回行ないＥｔＢｒを除去した。続いて、エタノール沈殿により、精製プラスミドを得た。

【００４６】中塩濃度緩衝液中で上記の精製したP7.5－E12をＨｉｎｄＩＩＩ により開裂した。この線状化した組換えベクターをトランスフェクション用に２５μｇ用意した。

【００４７】ｂ）トランスフェクション

遺伝子の導入はPerkesらのエレクトロポレーション法［Marion E. Perkus, Keith Limbach and Enzo Paoletti、J. Virol. 63, 3829−3836 (1989)］に準じて行った。すなわち、１７５cm² のカルチャーボトルに単層培養したウサギ腎臓由来細胞株ＲＫ１３細胞にワクシニアウイルス・リスター株をm.o.i. 5で感染させ、３７℃、５％ＣＯ₂ 下で１時間吸着させた後、トリプシンを用いて感染細胞を回収した。回収した細胞をＨｅＢＳバッファー（ｐＨ7.05）で２回洗浄し、ａ）でトランスフェクション用に調製したＤＮＡ２５μｇと共に０．８mlのＨｅＢＳバッファーに懸濁した。パルサーキュベット（バイオラッド社製）に細胞懸濁液を移し、この状態で１０分間、氷上にて冷却した。この後、バイオラッドジーンパルサーで２００Ｖ（Capacitance 、 960μＦ）のパルスを１回かけた。再度、氷上にて１０分間冷却し、細胞を２０mlの１０％ FCS−MEM に懸濁し、１７５cm² のカルチャーボトルで３７℃、５％ＣＯ₂ 存在下にて培養した。２４時間後、この培養物の凍結融解を３回繰り返し、ウイルスを回収した。

【００４８】回収したウイルスから組換えウイルスを赤血球吸着試験（ＨＡ試験）によって選択した。試験の方

法は以下のとおりである。9cmシャーレに単層培養したＲＫ１３細胞に６００プラーク／シャーレになるようにウイルスを接種し、２日間培養してプラーを形成させた。培養上清を除き、０．５％のニワトリ赤血球液を添加した。３７℃で１０分間インキュベートした後、プラークを観察し、赤血球を吸着しないプラーク（組換えウイルス候補株）を得た。

【００４９】次に、非Ａ非Ｂ型肝炎ウイルス構造蛋白質領域の遺伝子をプローブとして、先に得られた組換えウイルス候補株３４クローンのうち１２クローンのプラークハイブリダイゼーションを行い、候補株から、さらに遺伝子が組込まれた組換えウイルスを選択した。まず、3cmシャーレに単層培養したＲＫ－１３細胞に組換えウイルス候補株を２０～５０プラーク／シャーレになるように接種し、２日間培養してプラークを形成させた。形成したプラークの上にナイロンメンブラン（ハイボンドＮ、アマーシャム社製）をのせてプラークをメンブラン上に移し、０．５Ｎ　ＮａＯＨにより５分間処理してＤＮＡを変性させた後、 1M Tris-HCl(pH7.4)で中和し、さらに1.5M NaCl 、0.5M Tris-HCl(pH 7.4) で処理してＤＮＡをメンブランに吸着させた。メンブランを風乾したのち、３０５ nm の紫外線を５分間照射してＤＮＡをメンブランに固定した。ハイブリダイゼーションバッファーにこのメンブランを浸し、６５℃で１時間インキュベートした。さらに、ランダムプライムラベリング法を用いて^{32}Ｐで標識した非Ａ非Ｂ型肝炎ウイルス構造蛋白質領域の遺伝子を加えたハイブリダイゼーションバッファーにメンブランを移し、６５℃で一晩反応させ、プローブＤＮＡと組換えウイルスＤＮＡをハイブリダイズさせた。１×ＳＳＣ、 0.1%ＳＤＳ、６５℃で１０分間ずつ２回洗浄し、－７０℃でオートラジオグラフィーを行ない、陽性クローンを検出した。

【００５０】この結果、５クローンが陽性であり、それぞれクローンをＰ７．５－Ｅ１２／ＲＬＶ　＃７、８、９、１０、１１と命名した。

【００５１】実施例５

間接蛍光抗体法による発現の確認

単層培養したＲＫ－１３細胞を0.05%トリプシン・０．１mM　ＥＤＴＡ溶液で処理し単細胞にしたのちにＭＥＭ培養液（５％仔牛血清、0.22%炭酸水素ナトリウム）に５万個／mlになるように分散する。この細胞溶液にワクシニア親株のリスター株及びＰ７．５－Ｅ１２／ＲＬＶをm.o.i.＝0.1 になるように別々に接種する。ウイルスが接種された細胞を２０μｌ／穴で１２穴スライドグラスにのせ３７℃、５％CO_2 下で１晩培養する。培養したスライドグラスを蒸留水で１回洗浄し風乾後、－２０℃の５％アセトン・５０％メタノール混液に１５分間漬けて固定化する。固定後風乾し、ＰＢＳ（－）で５０倍に希釈した非Ａ非Ｂ型肝炎患者血清を２０μｌ／穴ずつのせて３７℃で４０分間反応させる。４０分間の反応後ＰＢＳ（－）で３回洗浄し、２５０倍にＰＢＳ（－）で希釈した抗ヒトＩｇＧ・ＦＩＴＣ標識（ヤギ）を２０μｌ／穴ずつのせてさらに３７℃で３０分間反応させる。反応終了後ＰＢＳ（－）で３回洗浄し蛍光顕微鏡で観察したところリスター株を感染させた細胞には蛍光が認められなかったがＰ７．５－Ｅ１２／ＲＬＶを感染させた細胞には強い特異蛍光が認められた（図２）。

【００５２】実施例６

免疫沈降法による発現の確認

ＲＫ－１３細胞を５％牛血清ＥＭＥＭ培地で１晩培養しモノレーヤーを形成させた。これにリスター株又はＰ７．５－Ｅ１２／ＲＬＶを感染させグルコースの代りに１０mMフルクトースを含むＥＭＥＭに１００μＣｉの^{3}Ｈ－グルコサミンを添加して１６時間培養した。正常人血清又は非Ａ非Ｂ型肝炎患者血清とプロテインＡセファロースとを用いた免疫沈降法で特異抗原蛋白質を沈降させＳＤＳ－ポリアクリルアミドゲル電気泳動法（ＳＤＳ－ＰＡＧＥ）で分析した。その結果Ｐ７．５－Ｅ１２／ＲＬＶ感染細胞を非Ａ非Ｂ型肝炎患者血清で免疫沈降したものだけにｇｐ３５の特異的なバンドが認められ発現していることが確認された（図３）。

【００５３】実施例７

Ｐ７．５－Ｅ１２／ＲＬＶ免疫ウサギ血清を用いた免疫沈降試験

Ｐ７．５－Ｅ１２／ＲＬＶを日本白色種ウサギ（１．５～２．０kg）の背部皮内に10^{8} ＰＦＵを接種し２カ月後に血清を採取した。

【００５４】一方、ブルースクリプトＫＳベクター（Stategene）のＴ７プロモーター下流に C10－E12 DNA を挿入しインビトロ・トランスクリプションにより C10－E12DNA に相補的なＲＮＡを合成した。この合成したＲＮＡをもちいてウサギレティキュロサイト抽出液中でインビトロ・トランスレーションを行い C10－E12 DNAでコードされるポリペプチドの試験管内合成を行なった。

【００５５】先に得られたウサギ免疫血清と合成されたポリペプチドとを用いて実施例６と同様に免疫沈降試験を行なった（図４）。

【００５６】この結果レーン２に示したようにＰ７．５－Ｅ１２／ＲＬＶ免疫ウサギの血清中には非Ａ非Ｂ型肝炎ウイルスの構造蛋白質領域をコードする C10－E12 DNA からつくられたポリプロテインと特異的に反応する抗体が産生されていることが確認できた。

【００５７】

【発明の効果】本発明により、非Ａ非Ｂ型肝炎ウイルス抗原蛋白質、特にエンベロープ蛋白質のような糖蛋白質の発現、産生が可能となり、従って、非Ａ非Ｂ型肝炎の治療又は予防のための、有効な免疫化を付与し得るワクチン抗原の提供を実現した。

【図面の簡単な説明】

【図１】この図は、組換えプラスミド及び組換えワクシ

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ニアウイルスの作製方法を示す。図中、ｐ２２は非Ａ非Ｂ型肝炎ウイルスゲノム上のコアをコードする遺伝子、ｇｐ３５はエンベロープをコードする遺伝子、およびｇｐ７５は非構造蛋白質をコードする遺伝子を表わす。

【図２】この図は、ワクシニアウイルス・リスター株（対照）又はＰ７．５－Ｅ１２／ＲＬＶ感染細胞を非Ａ非Ｂ型肝炎患者血清と反応させた後、ＦＩＴＣ標識抗ヒトＩｇＧ抗体（ヤギ）を用いて蛍光抗体染色した結果を示す写真である。

【図３】この図は、ワクシニアウイルス・リスター株（対照）又はＰ７．５－Ｅ１２／ＲＬＶ感染細胞と正常人血清又は非Ａ非Ｂ型肝炎患者血清との免疫沈降試験の結果を示す写真である。但し、

レーン１，３：リスター株感染細胞

レーン２，４：Ｐ７．５－Ｅ１２／ＲＬＶ感染細胞

レーン１，２：正常人血清

レーン３，４：非Ａ非Ｂ型肝炎患者血清

を示す。また、図中左端の数字は分子量マーカー（アマシャム社レインボウマーカー使用）を示す。

【図４】この図は、Ｐ７．５－Ｅ１２／ＲＬＶ免疫ウサギ血清または正常人血清と、非Ａ非Ｂ型肝炎ウイルスの構造蛋白領域をコードするＣ１０－Ｅ１２　ＤＮＡをもとにインビトロ・トランスレーションにより作られたポリプロテインとの免疫沈降試験の結果および合成ポリプロテインの電気泳動の結果を示す写真である。レーン１～５の説明は以下のとおりである。

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レーン１：インビトロ・トランスレーションをキャニン・ミクロゾーム膜存在下で行い、糖鎖の付加と合成された蛋白質のプロセシングを行い、得られた合成蛋白質と正常人血清との免疫沈降試験の結果を示す。正常人血清には抗体が存在しないため特異的な沈降は認められない。

レーン２：上記の合成蛋白質とｐ７．５－Ｅ１２／ＲＬＶ免疫ウサギ血清との免疫沈降試験の結果を示す。ウサギ血清にはｇｐ３５に対する抗体が存在するため３５ｋＤａの分子量のところに特異的なバンドが認められる。

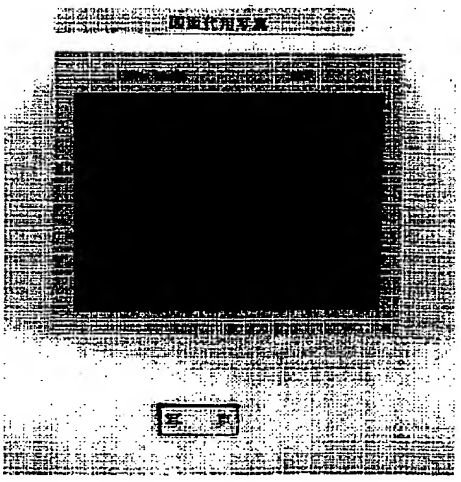
レーン３：上記の合成蛋白質の電気泳動の結果を示す。３５ｋＤａの分子量に相当するところにｇｐ３５の存在を示すバンドが認められる。

レーン４：キャニン・ミクロゾーム膜非存在下でインビトロ・トランスレーションを行って得られた合成蛋白質の電気泳動の結果を示す。合成された蛋白質は、プロセシングが行われていないので、ｇｐ３５の両端にｐ２２の一部およびｇｐ７５の一部が付加した蛋白質となっている。

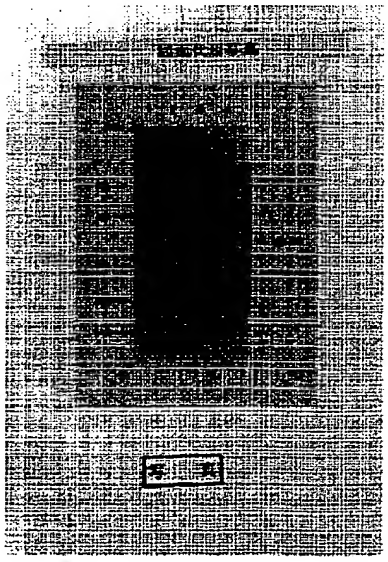
レーン５：インビトロ・トランスレーション時にＲＮＡを添加しなかった対照の電気泳動の結果を示す。蛋白質が合成されないためバンドが現れていない。

また、図中左端の数字は分子量マーカーを示す。

【図２】

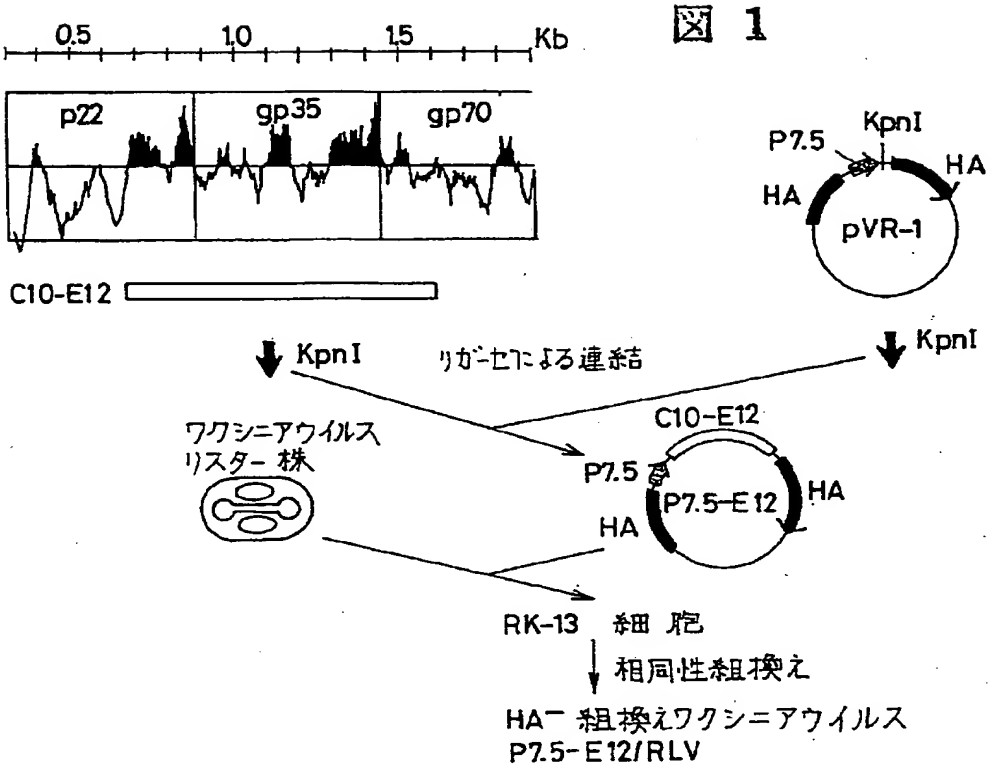


【図３】

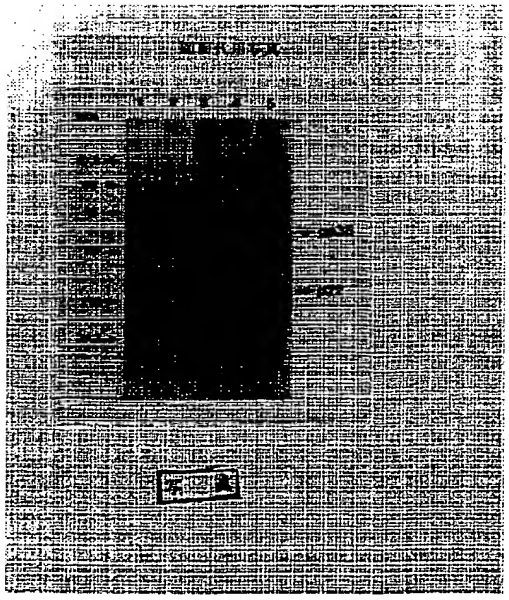


【図1】

図 1


0.5
1.0
1.5
Kb
p22
gp35
gp70
C10-E12
KpnI
P7.5
HA
HA
pVR-1
KpnI
リガーゼによる連結
KpnI
ワクシニアウイルス
リスター 株
C10-E12
P7.5
P7.5-E12
HA
HA
RK-13 細胞
相同性組換え
HA⁻ 組換えワクシニアウイルス
P7.5-E12/RLV

【図4】



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	15/39				
	15/51				
Ｃ１２Ｐ	21/00		Ｃ 8214－4Ｂ		
// Ｃ１２Ｎ	15/86				
(Ｃ１２Ｐ	21/00				
Ｃ１２Ｒ	1:91)				

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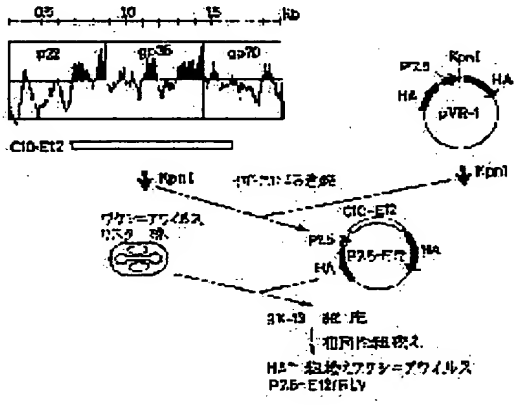
(54) MANIFESTATION OF NON-A, NON-B HEPATITIS VIRUS GENE USING RECOMBINANT VACCINIA VIRUS AND NON-A NON-B HEPATITIS VACCINE

(57)Abstract:
PURPOSE: To prepare a vaccine for preventing or improving non-A, non-B hepatitis by manifesting a non-A, non-B hepatitis virus antigen protein by using a recombinant vaccinia virus and using the resultant antigen protein.
CONSTITUTION: By homologeous recombination between a gene containing a base sequence coding a non-A, non-B hepatitis virus antigen protein (preferably envelope glucoprotein), a plasmid containing a virus promoter capable of manifesting the above-mentioned gene and a vaccinia virus gene (preferably hemagglutinin gene) not essential for propergation of a vaccinia virus, preferably plasmid P7.5-E12 and the vaccinia virus, a recombinant vaccinia virus (preferably P7.5-E12/RLV) containing an inserted gene coding the non-A, non-B hepatitis virus antigen protein is obtained. The objective vaccine is prepared by using the antigen protein manifested by using the above-mentioned virus or an attenuated or inactivated recombinant vaccinia virus.



LEGAL STATUS

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[Date of final disposal for application]

[Patent number]

[Date of registration]

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[Date of requesting appeal against examiner's decision of rejection]

[Date of extinction of right]

CLAIMS

[Claim(s)]
[Claim 1] The plasmid containing the vaccinia-virus gene which is not indispensable because of propagation of the gene containing the base sequence which carries out the code of the non-A-non-B-hepatitis virus-antigen protein, the virus promoting agent who may make this gene discover, and the vaccinia virus.
[Claim 2] The plasmid according to claim 1 whose aforementioned antigen protein is a virus structural protein.
[Claim 3] The plasmid according to claim 2 whose aforementioned structural protein is envelope glycoprotein.
[Claim 4] The plasmid according to claim 1 whose aforementioned virus promoting agent is the promoting agent of the vaccinia-virus origin.
[Claim 5] The plasmid according to claim 1 whose aforementioned vaccinia-virus gene is a hemagglutinin gene.
[Claim 6] The plasmid according to claim 1 to which 1 or more sets of the gene containing the base sequence which carries out the code of the aforementioned antigen protein, and the aforementioned virus promoting agent are characterized by being inserted into the aforementioned vaccinia-virus gene.
[Claim 7] Plasmid P7.5-E12.
[Claim 8] Recombination vaccinia virus obtained by homology recombination of a plasmid given in any 1 term of claims 1-7 and the vaccinia virus.
[Claim 9] Recombination vaccinia-virus P7.5-E12/RLV.
[Claim 10] the phase of being the manufacture technique of the recombination vaccinia virus according to claim 8 or 9, line-izing the plasmid of a publication restrictively in any 1 term of claims 1-7, carrying out a transfection to the animal cell with which the vaccinia virus is infected, and performing homology recombination — and — The technique of screening the recombination virus in which the gene containing the base sequence which carries out the code of the non-A-non-B-hepatitis virus-antigen protein is inserted, and including the phase to collect.
[Claim 11] Technique according to claim 10 the vaccinia virus is a vaccinia-virus Lister stock.
[Claim 12] Technique according to claim 10 the aforementioned animal cell is lagomorph kidney passage cell RK-13.
[Claim 13] The manufacture technique of the manifestation non-A-non-B-hepatitis virus-antigen protein which consists of the recombination vaccinia virus according to claim 8 or 9 being infected with an animal cell, making the gene containing the base sequence which cultivates this cell and carries out the code of the non-A-non-B-hepatitis virus-antigen protein discover, and collecting manifestation protein.
[Claim 14] Technique according to claim 13 the aforementioned animal cell is lagomorph kidney passage cell RK-13.
[Claim 15] Manifestation non-A-non-B-hepatitis virus-antigen protein which is obtained by technique according to claim 13 or 14 and which has sugar.
[Claim 16] The vaccine for treating or preventing non-A non-B hepatitis containing the manifestation non-A-non-B-hepatitis virus-antigen protein which has sugar according to claim 15.
[Claim 17] The vaccine for treating or preventing non-A non-B hepatitis containing the recombination vaccinia virus according to claim 8 or 9 which carried out the attenuation.
[Claim 18] The vaccine for treating or preventing non-A non-B hepatitis containing the recombination vaccinia virus according to claim 8 or 9 which carried out the inactivation.

DETAILED DESCRIPTION

[Detailed Description of the Invention]
[0001]
[Field of the Invention] this invention relates to a manifestation of the non-A-non-B-hepatitis virus-antigen protein which uses the recombination vaccinia virus, and the application to a vaccine.
[0002]
[Description of the Prior Art] After sideration, non-A non-B hepatitis is an infectious hepatitis of virus nature, many become chronic, also socially, it is a big problem from shifting to high rate to liver cirrhosis and a hepatic carcinoma, and the prophylaxis is needed. There are two kinds of grasping the technique and the infection status of using a vaccine for a prevention of the virus nature morbus, and severing the route. Among these, the kit required to grasp the infection status for a diagnosis is ********. It is already developed by M.Houghton et al. (****** official report of No. 500880 [two to]), ** et al. (Japanese Patent Application No. 180889 [two to]), and infection by transfusion can be considerably prevented now at high rate. However, a non-A-non-B-hepatitis patient's abbreviation moiety (about 500,000 persons/(year)) is an infection person who does not mind transfusion, there is no effective means to prevent these infection now, and desire of the development of a prevention vaccine is carried out.
[0003] About this kind of vaccine, M.Houghton et al. has already indicated about the vaccine containing the immunogenicity polypeptide which contains a Homo-sapiens hepatitis C virus (HCV) epitope in the above-mentioned official report, the vaccine containing inactivation HCV, and the vaccine containing an attenuation HCV.
[0004]
[Problem(s) to be Solved by the Invention] Usually, although the virus by which the inactivation was carried out with medicines, such as formalin, itself is used for a vaccine in many cases, a non-A-non-B-hepatitis virus is not yet separated, and it is in vitro. Since there is also no virus propagation system, this virus cannot be obtained in large quantities. Therefore, a non-A-non-B-hepatitis virogene is made to discover by Escherichia coli, yeast, the animal cell, etc., and obtaining a vaccine antigen is suggested (****** official report of No. 500880 [two to]).
[0005] Although the protein which has sugar like viral-envelope protein generally excels [immunogenicity] in the immunization highly compared with the protein without it, about the effective discovered method of such glycoprotein, and its acquisition, M.Houghton et al. is not taught at all. The manifestation system which used the animal cell for making glycoprotein like viral-envelope protein discover in the type which the sugar chain added can be considered. However, it is quite difficult to obtain manifestation protein only atmg several //l.] luminous efficacy generally, but to raise purity with the manifestation system using the animal cell even to the usable degree of refining as a vaccine.
[0006] By using zealously the recombination vaccinia virus obtained by the homology recombination between the recombination plasmids and vaccinia virus containing the gene and virus promoting agent containing the base sequence which carries out the code of the non-A-non-B-hepatitis virus-antigen protein into a vaccinia-virus gene as a result of a research, this invention persons found out that the non-A-non-B-hepatitis virus-antigen protein with sugar might be discovered, and completed this invention.
[0007] this invention aims at offering the manifestation system of non-A-non-B-hepatitis virus-antigen protein which uses the recombination vaccinia virus.
[0008] this invention aims at offering again this antigen protein near the nature type or nature type obtained through this manifestation system.
[0009] this invention aims at the application to the vaccine of an attenuation or the inactivation rec mbination vaccinia virus, and this antigen protein further.
[0010] Since immunity is given when a virus increases by the inoculation site when using the recombination vaccinia virus as a vaccine, it is good at very little use, and refining is also easy.
[0011]
[Means for Solving the Problem] this invention offers the plasmid containing the vaccinia-virus gene which is not indispensable because of propagation of the virus promoting agent who may make the gene containing the base sequence which carries out the code of the non-A-non-B-hepatitis virus-antigen protein, and this gene discover, and the vaccinia virus.
[0012] This plasmid is the frame indispensable in rearranging by homology recombination between vaccinia virus and producing the vaccinia virus.
[0013] In the gene containing the base sequence which carries out the code of the non-A-non-B-hepatitis virus-antigen protein, although any of the non-structural protein of a non-A-non-B-hepatitis virus or a

structural protein are sufficient as this antigen protein by which a code is carried out, in order to realize an efficient immunization, the protein which constitutes a structural protein especially an envelope, and a core is desirable. An envelope is structure which constitutes an animal-viruses jacket, and contains the glycoprotein structure made by the lipid-bilayer layer similar to a cytoplasmic membrane, and the virogene. The end is embedded into this layer and the other end has projected this glycoprotein structure outside in the shape of a salient (called a spike). Moreover, a core is also called internal capsid and constitutes the husks which consist of the protein which wraps a viral genome. Although this antigen protein does not necessarily need sugar, in order to raise an immunogenicity, it is desirable that it is glycoprotein and the characteristic feature of this invention which enables an efficient manifestation of glycoprotein in this case may fully be demonstrated. In the below-mentioned example, the gene which carries out the code of the envelope antigen glycoprotein gp35 of a non-A-non-B-hepatitis virus is actually included in a plasmid, and the manifestation is realized.

[0014] The virus promoting agent will not ask the modality, if the gene containing the base sequence which carries out the code of the aforementioned antigen protein may be made to discover. As such promoting agent, although the vaccinia-virus origin promoting agent (7.5kD protein, ATI promoting agent), the cytomegalovirus promoting agent, etc. are mentioned, in order to make this gene expression within the recombination vaccinia virus perform effectively, the vaccinia-virus origin promoting agent is desirable.

[0015] A vaccinia-virus gene has desirable things other than the gene concerning the propagation system of this virus, and a hemagglutinin (HA) gene may be suitably used especially in respect of screening of a recombination virus.

[0016] Moreover, it is suitable that arrangement of these genes and the promoting agent takes the gestalt by which both were inserted in the interior of a vaccinia-virus gene so that the gene which generally contains the base sequence which carries out the code of the non-A-non-B-hepatitis virus-antigen protein may be arranged on the virus promoting agent's lower stream of a river.

[0017] Plasmid P7.5-E12 by the embodiment of this invention can use it suitably as this invention plasmid, and this specific plasmid is also contained within the limits of this invention. The plasmid of this instantiation isolates HA gene from a vaccinia-virus WR stock first, as shown in the below-mentioned example. A cloning can be carried out to pUC18, 7.5kD protein promoting agent of the vaccinia-virus origin can be inserted in NruI site of this HA gene, and it can produce by inserting the gene containing the base sequence which carries out the code of the non-A-non-B-hepatitis virus-antigen protein to the lower stream of a river (refer to the drawing 1).

[0018] this invention offers again the recombination vaccinia virus obtained by homology recombination of an above-mentioned recombination plasmid and the vaccinia virus.

[0019] this invention recombination vaccinia virus can line-ize the above-mentioned recombination plasmid of this invention restrictively, can screen the recombination virus in which the phase of carrying out a transfection to the animal cell with which the vaccinia virus is infected, and performing homology recombination, and the gene which carries out the code of the non-A-non-B-hepatitis virus-antigen protein are inserted, and can manufacture it by the technique containing the phase of collecting them. This manufacture technique is also included within the limits of this invention.

[0020] A vaccinia-virus Lister stock may be suitably used as vaccinia virus by the embodiment of this invention. Moreover, as an animal cell for infecting this virus, the mammalian cell is suitable, for example, a lagomorph kidney passage cell (RK-13 cell) can be mentioned.

[0021] Recombination vaccinia-virus P7.5-E12/RLV by the embodiment of this invention can use it suitably, and this is also contained within the limits of this invention. the gene to which line-ized plasmid p7.5-E12/RLV carries out the code of the HA (hemagglutinin) protein in a vaccinia-virus gene in this recombination field virus — it is mostly inserted in the center For this reason, normal hemagglutinin protein is not produced. Moreover, since the inserted non-A-non-B-hepatitis virogene has a start codon ATG in the fraction of the head, mRNA is newly compounded from here and protein synthesis is performed.

[0022] Screening of the recombination vaccinia virus acquires the recombination virus candidate stock of HA (-) first by hemadsorption examination (HA examination) (Shida, H., virology 150:451-462, and 1986), and can be carried out by the technique of detecting an electropositive clone by the plaque hybridization which uses as a probe the nucleotide sequence which carries out the code of the peptide which subsequently constitutes non-A-non-B-hepatitis virus-antigen protein.

[0023] Rearrange, the vaccinia virus is infected with an animal cell, this invention makes the gene containing the base sequence which was produced still as mentioned above and which cultivates this cell and carries out the code of the non-A-non-B-hepatitis virus-antigen protein discover, and the manufacture technique of the manifestation non-A-non-B-hepatitis virus-antigen protein which consists of collecting manifestation protein is offered.

[0024] The mammalian cell is suitable and, as for an animal cell, a lagomorph kidney passage cell (RK-13 cell) may be used especially suitably. Moreover, a culture condition is determined depending on the animal cell to use, and the culture medium which can be increased, incubation temperature, incubation time, etc. are chosen suitably. For example, in the aforementioned P7.5-E12/RLV, the cow blood serum inclusion EMEM culture medium which contains a fructose instead of a glucose may be used.

[0025] an antibody [as opposed to this antigen protein in a manifestation of non-A-non-B-hepatitis virus-antigen protein] — or it may be checked by the immunological technique of the common use like the immunoprecipitation method using the non-A-non-B-hepatitis patient blood serum containing it, and an indirect fluorescent antibody technique (the drawing 2 and drawing 3)

[0026] this invention offers the manifestation non-A-non-B-hepatitis virus-antigen protein which has especially sugar among the non-A-non-B-hepatitis virus-antigen protein again discovered by doing in this way.
[0027] The manifestation of glycoprotein made this gene expression possible for the first time by invention of the manifestation system which uses the recombination vaccinia virus containing the gene containing the base sequence which is not yet realized in a non-A-non-B-hepatitis virus relation field, and carries out the code of this antigen glycoprotein.
[0028] The manifestation non-A-non-B-hepatitis virus-antigen protein which has "sugar" means including both nature type non-A-non-B-hepatitis virus-antigen glycoprotein and its analog among this specification. moreover, the thing for which the analog of this nature type antigen glycoprotein prevents the irruption to the animal inside of the body of a non-A-non-B-hepatitis virus — or the alteration field of the domain which can produce the antibody to the non-A-non-B-hepatitis virus which can eradicate this virus infected with the animal is meant
[0029] this invention offers the vaccine for treating or preventing non-A non-B hepatitis containing the manifestation non-A-non-B-hepatitis virus-antigen protein which has the further above-mentioned sugar.
[0030] Again, the attenuation of this invention was carried out, or it offers the vaccine for treating or preventing the non-A non-B hepatitis which carried out the inactivation and which rearranges and contains the vaccinia virus.
[0031] The vaccinia virus is a kind of the poxvirus group used to build the Homo sapiens's variola immunity. After proliferating the recombination vaccinia virus in the cell of an animal, an organization, the organum, etc., it can consider as an attenuated metaplasia vaccine, or virulence can be weakened, an inactivation can be carried out with formaldehyde, and it can consider as a killed vaccine. It is actually proved by the below-mentioned example 7 by inoculating recombination vaccinia-virus P7.5-E12/RLV in a lagomorph regions-of-back hide that the antibody to non-A-non-B-hepatitis virus-antigen protein is produced in a lagomorph blood serum (drawing 4).
[0032] the gestalt of the adjuvant vaccine, like generally, an immunogen is contained in a water in oil emulsion, the vaccine of this invention is adsorbed on inorganic gels, such as an aluminum hydroxide and an aluminium phosphate, or the organic adjuvant of common use is included — or although used with the solution gestalt in liquid solvents, such as a physiological saline and a glycerol, it is not limited to this Moreover, as an application of a vaccine, inoculation is desirable, and a medicine is prescribed for the patient in the amount from which the prevention effect or curative effect made into the purpose is acquired, and it depends for the amount on the age of an individual, weight, a symptom, etc.
[0033]
[Example] Although this invention is explained still in detail, this invention is not limited to these examples by the following examples, unless the summary of this invention is changed.
[0034] Example The cloning vaccinia-virus WR stock of the hemagglutinin (HA) gene of one vaccinia virus is refined by the cane-sugar density gradient centrifugation [Joklik.W.K, Virology, 18, and 9-18 (1962)]. a 50mM Tris-HCl (pH 7.4) buffer (it EDTAs 1mM —) It suspends during 0.5% inclusion [sodium-dodecyl-sulfate], and is pro tee ****** K. After incubating at 37 degrees C in addition overnight so that it may become 250-1000microg [/ml] concentration, TE buffer extracted 3 times with bottom phenol [of a saturation]:chloroform (1:1) liquid, ethanol precipitation was performed, and the virus DNA was obtained. It is HindIII in the Nakashio concentration buffer solution about this DNA. It digested and Hind IIIA fragment of about 50 kbs was obtained by the agarose gel electrophoresis. This Hind IIIA fragment was digested by SalI in the high salt concentration buffer solution, and the Hind III-SalI fragment of about 1.8 kbp located in 3' terminal of Hind IIIA fragment was isolated by the agarose gel electrophoresis.
[0035] On the other hand, it is HindIII in the Nakashio concentration buffer solution about a plasmid pUC13. It continued, and in the high salt concentration buffer solution, it digested by SalI and line-ized. this line — the-izing plasmid was isolated by the agarose gel electrophoresis
[0036] the aforementioned Hind III-SalI fragment and a line — a-izing plasmid — the inside of the ligation buffer solution — T4 ligase — connecting — this reaction mixture — using — Escherichia coli JM103 The transformation was carried out. Plasmids were collected from each transformant with the alkali extraction method. Furthermore analysis by the restriction enzyme was performed, the plasmid which has the gene configuration made into the purpose was chosen, and this was named pHA13.
[0037] Example The vector for recombination for incorporating the foreign gene connected with 7.5kD protein promoting agent and its lower stream of a river of the production vaccinia virus of the multi-vector for recombination using 7.5kD protein promoting agent of two vaccinia virus in the genome HA gene of the vaccinia virus was produced by the following technique.
[0038] First, from the vaccinia-virus WR stock, it extracted like the technique which showed DNA in the example 1, and 7.5kD protein promoting agent was isolated by Venkatesan's et al. technique [Venkatesan & B.Moss, J.Virol.33, and 738-745 (1981)]. That is, DNA of extracted WR stock was digested in SalI, and the cloning of the DNA fragment of obtained 0.9kbps was carried out to SalI site of pUC18. Furthermore, this plasmid was digested by RsaI and HincII, and the RsaI-HincII fragment of 0.26 kbp including 7.5kD protein promoting agent was obtained. The cloning of this DNA fragment was carried out to pUC18 (p 7.5-18).
[0039] Next, 7.5kD protein promoting agent who isolated by the above technique was inserted in NruI site of HA gene which carried out the cloning in the example 1 by the following technique.
[0040] They are EcoRI and HindIII in the Nakashio concentration buffer solution about plasmid p 7.5-18. By digesting, it is about 0.29 kbp. 7.5k promoter gene was started and it isolated by the agarose gel

electrophoresis. The isolated gene fragment was made to react to the bottom of dTTP, dCTP, dATP, and dGTP presence with Klenow fragment in the nick-translation buffer solution, and the terminal was made smooth.
[0041] On the other hand, plasmid pHA-13 were digested and line-ized in NruI buffer solution. The gene fragment which this line-ized plasmid and the above smoothed is connected by T4 ligase in the ligation buffer solution, and it is Escherichia coli JM109. The transformation was carried out. 7.5kD promoting agent (P7.5) was inserted in the orientation and the same direction of HA gene as a result of analysis according the plasmid obtained from the transformant] to a restriction enzyme. This was nam d pVR-1 (drawing 1).
[0042] Example The recombination vector used in order to incorporate the gene of the envelope field of the production non-A-non-B-hepatitis virus of 3 recombination plasmid into HA gene of the genome of the vaccinia virus was produced by the following technique.
[0043] Clone C10-E12 (fine ********** of No. 3444) (Japanese Patent Application No. 413844 [two to]) with the field which carries out the code of the structural protein of a non-A-non-B-hepatitis virus was digested in KpnI in the low-salt concentration buffer solution, the gene fragment of about 1.0 kbps was isolated by the agarose gel electrophoresis, and the gene fragment was obtained (drawing 1).
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[0045] Example Plasmid P7.5-E12 of 150microg was extracted from the Escherichia coli cultivated by 200ml culture medium using the manufacture DIAGEN plasmid kit (product made from DIAGEN) of DNA used for the production a transfection of 4 recombination vaccinia virus. This plasmid was refined by CsCl density gradient centrifugation. That is, they are 10 degrees C and 55,000rpm about that with which melted the plasmid in rho= 1.47g [/ml] CsCl liquid (EtBr inclusion), and the quick seal tube (made in Beckmann, an ultra clearance, 13x51mm) was filled up. The at-long-intervals core was carried out at 15:00. (VTi 65.2 rotor, Beckmann ultracentrifuge) . After centrifugal and closed circular The bands of plasmid DNA were collected and deed EtBr was removed for the isopropanol extraction 3 times. Then, the refining plasmid was obtained by ethanol precipitation.
[0046] It is HindIII about P7.5-E12 which the above refined in the Nakashio concentration buffer solution. It clove. 25microg readiness of this line-ized recombination vector was carried out for transfections.
[0047] b) The introduction of a transfection gene was performed according to Perkes's et al. electroporation method [Marion E.Perkus, Keith Limbach and Enzo Paoletti, J.Virol.63, 3829-3836 (1989)]. Namely, 175cm2 A vaccinia-virus Lister stock is infected with lagomorph ren origin cell-strain RK13 cell which carried out the monolayer culture to the culture bottle by m.o.i.5, and they are 37 degrees C and 5%CO2. After making it adsorb in the bottom for 1 hour, infected cells were collected using the trypsin. HeBS buffer (pH7.05) washed the collected cell twice, and it suspended in 0.8ml HeBS buffer with DNA25microg prepared to transfections by a. The cell suspension was moved to the pulsar cuvette (Bio-Rad make), and it cooled for 10 minutes in this status in Hikami. Then, the pulse of 200V (Capacitance and 960 micro F) was applied once in the ********** pulsar. It cools for 10 minutes again in Hikami, and is 20ml 10% FCS-MEM about a cell. It suspends and is 2 175cm. They are 37 degrees C and 5%CO2 with a culture bottle. It cultivated under presence. The freeze thawing of this culture was repeated 3 times 24 hours after, and viruses were collected.
[0048] It rearranged from the collected virus and the virus was chosen by hemadsorption examination (HA examination). The experimental technique is as follows. The virus was inoculated so that it might become 600 plaques / laboratory dish into RK13 cell which carried out the monolayer culture to 9cm laboratory dish, and it cultivated for two days, and ****** was made to form. Except for the culture supernatant, 0.5% of fowl erythrocyte liquid was added. After incubating for 10 minutes at 37 degrees C, the plaque was observed and the plaque (recombination virus candidate stock) which does not adsorb an erythrocyte was obtained.
[0049] Next, the recombination [which was obtained previously] virus in which it rearranges, the plaque hybridization of 12 clone is performed among virus candidate stock 34 clones, and the gene was further included from the candidate stock was chosen, having used the gene of a non-A-non-B-hepatitis virus structural-protein field as the probe. First, it rearranged into RK-13 cell which carried out the monolayer culture to 3cm laboratory dish, the virus candidate stock was inoculated so that it might become 20 - 50 plaque / laboratory dish, and it was cultivated for two days, and the plaque was made to form. the formed plaque top — a nylon membrane (high bond N, product made from Amersham) — carrying — a plaque — a membrane top — moving — 0.5N 1M Tris-HCl (pH7.4) after having processed for 5 minutes by NaOH and making DNA denaturalize — neutralizing — further — 1.5M NaCl and 0.5M Tris-HCl (pH 7.4) It processed and DNA was made to stick to a membrane. 305 nm aft r air-drying a membrane Ultraviolet rays were irradiated for 5 minutes and DNA was fixed to the membrane. This membrane was dipped in the hybridization buffer and it incubated at 65 degrees C for 1 hour. Furthermore, moved the membrane to the hybridization buffer which added the gene of the non-A-non-B-hepatitis virus structural-prot in field which carried out the indicator by 32P using the random prime labeling method, and it was made to react at 65 degrees C overnight, and rearranged with probe DNA, and the virus DNA was made to hybridize. it washed twice every / during 10 minutes] at 1xSSC, 0.1% SDS, and 65 degrees C, autoradiography was performed at -70 degrees C, and the electropositive clone was detected

[0050] Consequently, five clones are positivities and it is P7.5-E12/RLV about a clone, respectively. It was named #7, and 8, 9, 10 and 11.
[0051] They are 0.05% trypsin and 0.1mM about RK-13 cell in which the manifestation by example 5 indirect fluorescent antibody technique carried out the authentication monolayer culture. After processing with EDTA solution and making it a single cell, it distributes so that it may become [ml] MEM culture medium (5% calf serum, 0.22% sodium hydrogencarbonate) in 50,000 pieces /. They are the Lister stock of a vaccinia parent strain, and P7.5-E12/RLV to this cell solution m.o.i.=0.1 It inoculates separately so that it may become. The cell into which the virus was inoculated is put on 12 hole slide glass in 20microl / hole, and they are 37 degrees C and 5%CO2. One evening is cultivated in the bottom. Distilled water washes the cultivated slide glass once, and after air-drying, it soaks in a methanol mixture for 15 minutes -20-degree C 5% acetone and 50%, and fixes. Are air-dry after fixation, and the non-A-non-B-hepatitis patient blood serum diluted with PBS (-) 50 times is carried 20microl / hole every, and is made to react for 40 minutes at 37 degrees C. It washes 3 times by PBS after a reaction for 40 minutes (-), and anti-Homo-sapiens IgG and FITC indicator (goat) diluted with PBS (-) 250 times are carried 20microl / hole every, and is made to react for 30 minutes at 37 more degrees C. Although fluorescence was not accepted in the cell with which the Lister stock was infected when it washed 3 times by PBS after a reaction end (-) and having been observed with the fluorescence microscope, unique fluorescence strong against the cell with which P7.5-E12/RLV was infected accepted (drawing 2).
[0052] One evening of authentication RK-13 cells of the manifestation by the example 6 immunoprecipitation method was cultivated by the cow blood serum EMEM culture medium 5%, and the mono-layer was made to form. It is 100microcurie to EMEM which the Lister stock or P7.5-E12/RLV is infected with this, and contains 10mM fructose instead of a glucose. The 3H-glucosamine was added and it cultivated for 16 hours. Unique antigen protein was made to sediment by the immunoprecipitation method using a normal people blood serum or a non-A-non-B-hepatitis patient blood serum, and protein A sepharose, and it analyzed by the SDS-polyacrylamide-gel-electrophoresis method (SDS-PAGE). It was checked that the specific band of gp35 is accepted only in what, as a result, carried out the immunoprecipitation of P7.5-E12 / the RLV infected cell by the non-A-non-B-hepatitis patient blood serum, and it is discovered (drawing 3).
[0053] 108 PFU was inoculated for immunoprecipitation examination P7.5-E12/RLV using example 7P7.5-E12 / RLV immunity lagomorph blood serum in the regions-of-back hide of the Japanese white kind lagomorph (1.5-2.0kg), and the blood serum was extracted two months after.
[0054] On the other hand, it is on T7 promoting-agent lower stream of a river of a blue script KS vector (Strategene). C10-E12 DNA It inserts and is an in vitro transcription. C10-E12DNA Complementary RNA was compounded. It is with this compound RNA and the in vitro translation is performed in a lagomorph ********** site extract. The synthesis in a test tube of the polypeptide by which a code is carried out by C10-E12 DNA was performed.
[0055] The immunoprecipitation examination was performed like the example 6 using the polypeptide compounded with the lagomorph immune serum obtained previously (drawing 4).
[0056] as shown in the lane 2 as a result, into the blood serum of P7.5-E12 / RLV immunity lagomorph, the code of the structural-protein field of a non-A-non-B-hepatitis virus is carried out C10-E12 DNA **** — it has checked that the antibody which reacts specifically with the built polypropylene theine was produced
[0057]
[Effect of the Invention] Offer of the vaccine antigen which a manifestation of glycoprotein like non-A-non-B-hepatitis virus-antigen protein, especially envelope protein and production of are attained, therefore can give the effective immunization for the treatment of non-A non-B hepatitis or a prevention by this invention was realized.

[Translation done.]

TECHNICAL FIELD

[Field of the Invention] this invention relates to a manifestation of the non-A-non-B-hepatitis virus-antigen protein which uses the recombination vaccinia virus, and the application to a vaccine.

[Translation done.]

PRIOR ART

[Description of the Prior Art] After sideration, non-A non-B hepatitis is an infectious hepatitis of virus nature, many become chronic, also socially, it is a big problem from shifting to high rate to liver cirrhosis and a hepatic carcinoma, and the prophylaxis is needed. There are two kinds of grasping the technique and the infection status of using a vaccine for a prevention of the virus nature morbus, and severing the route. Among these, the kit required to grasp the infection status for a diagnosis is ********. It is already developed by M.Houghton et al. (****** official report of No. 500880 [two to]), ** et al. (Japanese Patent Application No. 180889 [two to]), and infection by transfusion can be considerably prevented now at high rate. However, a non-A-non-B-hepatitis patient's abbreviation moiety (about 500,000 persons/(year)) is an infection person who does not mind transfusion, there is no effective means to prevent these infection now, and desire of the development of a prevention vaccine is carried out.
[0003] About this kind of vaccine, M.Houghton et al. has already indicated about the vaccine containing the immunogenicity polypeptide which contains a Homo-sapiens hepatitis C virus (HCV) epitope in the above-mentioned official report, the vaccine containing inactivation HCV, and the vaccine containing an attenuation HCV.

[Translation done.]

EFFECT OF THE INVENTION

[Effect of the Invention] Offer of the vaccine antigen which a manifestation of glycoprotein like non-A-non-B-hepatitis virus-antigen protein, especially envelope protein and production of are attained, therefore can give the effective immunization for the treatment of non-A non-B hepatitis or a prevention by this invention was realized.

[Translation done.]

TECHNICAL PROBLEM

[Problem(s) to be Solved by the Invention] Usually, although the virus by which the inactivation was carried out with medicines, such as formalin, itself is used for a vaccine in many cases, a non-A-non-B-hepatitis virus is not yet separated, and it is in vitro. Since there is also no virus propagation system, this virus cannot be obtained in large quantities. Therefore, a non-A-non-B-hepatitis virogene is made to discover by Escherichia coli, yeast, the animal cell, etc., and obtaining a vaccine antigen is suggested (****** official report of No. 500880 [two to]).

[0005] Although the protein which has sugar like viral-envelope protein generally excels [immunogenicity] in the immunization highly compared with the protein without it, about the effective discovered method of such glycoprotein, and its acquisition, M.Houghton et al. is not taught at all. The manifestation system which used the animal cell for making glycoprotein like viral-envelope protein discover in the type which the sugar chain added can be considered. However, it is quite difficult to obtain manifestation protein only atmg several //l.] luminous efficacy generally, but to raise purity with the manifestation system using the animal cell even to the usable degree of refining as a vaccine.

[0006] By using zealously the recombination vaccinia virus obtained by the homology recombination between the recombination plasmids and vaccinia virus containing the gene and virus promoting agent containing the base sequence which carries out the code of the non-A-non-B-hepatitis virus-antigen protein into a vaccinia-virus gene as a result of a research, this invention persons found out that the non-A-non-B-hepatitis virus-antigen protein with sugar might be discovered, and completed this invention.

[0007] this invention aims at offering the manifestation system of non-A-non-B-hepatitis virus-antigen protein which uses the recombination vaccinia virus.

[0008] this invention aims at offering again this antigen protein near the nature type or nature type obtained through this manifestation system.

[0009] this invention aims at the application to the vaccine of an attenuation or the inactivation recombination vaccinia virus, and this antigen protein further.

[0010] Since immunity is given when a virus increases by the inoculation site when using the recombination vaccinia virus as a vaccine, it is good at very little use, and refining is also easy.

[Translation done.]

MEANS

[Means for Solving the Problem] this invention offers the plasmid containing the vaccinia-virus gene which is not indispensable because of propagation of the virus promoting agent who may make the gene containing the base sequence which carries out the code of the non-A-non-B-hepatitis virus-antigen protein, and this gene discover, and the vaccinia virus.
[0012] This plasmid is the frame indispensable in rearranging by homology recombination between vaccinia virus and producing the vaccinia virus.
[0013] In the gene containing the base sequence which carries out the code of the non-A-non-B-hepatitis virus-antigen protein, although any of the non-structural protein of a non-A-non-B-hepatitis virus or a structural protein are sufficient as this antigen protein by which a code is carried out, in order to realize an efficient immunization, the protein which constitutes a structural protein especially an envelope, and a core is desirable. An envelope is structure which constitutes an animal-viruses jacket, and contains the glycoprotein structure made by the lipid-bilayer layer similar to a cytoplasmic membrane, and the virogene. The end is embedded into this layer and the other end has projected this glycoprotein structure outside in the shape of a salient (called a spike). Moreover, a core is also called internal capsid and constitutes the husks which consist of the protein which wraps a viral genome. Although this antigen protein does not necessarily need sugar, in order to raise an immunogenicity, it is desirable that it is glycoprotein and the characteristic feature of this invention which enables an efficient manifestation of glycoprotein in this case may fully be demonstrated. In the below-mentioned example, the gene which carries out the code of the envelope antigen glycoprotein gp35 of a non-A-non-B-hepatitis virus is actually included in a plasmid, and the manifestation is realized.
[0014] The virus promoting agent will not ask the modality, if the gene containing the base sequence which carries out the code of the aforementioned antigen protein may be made to discover. As such promoting agent, although the vaccinia-virus origin promoting agent (7.5kD protein, ATI promoting agent), the cytomegalovirus promoting agent, etc. are mentioned, in order to make this gene expression within the recombination vaccinia virus perform effectively, the vaccinia-virus origin promoting agent is desirable.
[0015] A vaccinia-virus gene has desirable things other than the gene concerning the propagation system of this virus, and a hemagglutinin (HA) gene may be suitably used especially in respect of screening of a recombination virus.
[0016] Moreover, it is suitable that arrangement of these genes and the promoting agent takes the gestalt by which both were inserted in the interior of a vaccinia-virus gene so that the gene which generally contains the base sequence which carries out the code of the non-A-non-B-hepatitis virus-antigen protein may be arranged on the virus promoting agent's lower stream of a river.
[0017] Plasmid P7.5-E12 by the embodiment of this invention can use it suitably as this invention plasmid, and this specific plasmid is also contained within the limits of this invention. The plasmid of this instantiation isolates HA gene from a vaccinia-virus WR stock first, as shown in the below-mentioned example. A cloning can be carried out to pUC18, 7.5kD protein promoting agent of the vaccinia-virus origin can be inserted in NruI site of this HA gene, and it can produce by inserting the gene containing the base sequence which carries out the code of the non-A-non-B-hepatitis virus-antigen protein to the lower stream of a river (ref r to the drawing 1).
[0018] this invention offers again the recombination vaccinia virus obtained by homology recombination of an above-mentioned recombination plasmid and the vaccinia virus.
[0019] this invention recombination vaccinia virus can line-ize the above-mentioned recombination plasmid of this invention restrictively, can screen the recombination virus in which the phase of carrying out a transfection to the animal cell with which the vaccinia virus is infected, and performing homology recombination, and the gene which carries out the code of the non-A-non-B-hepatitis virus-antigen protein are inserted, and can manufacture it by the technique containing the phase of collecting them. This manufacture technique is also included within the limits of this invention.
[0020] A vaccinia-virus Lister stock may be suitably used as vaccinia virus by the embodiment of this invention. M reover, as an animal cell for infecting this virus, the mammalian cell is suitable, for example, a lagomorph kidney passage cell (RK-13 cell) can be mentioned.
[0021] Recombination vaccinia-virus P7.5-E12/RLV by the embodiment of this invention can use it suitably, and this is also contained within the limits of this invention. the gene to which line-ized plasmid p7.5-E12/RLV carries out the code of the HA (hemagglutinin) protein in a vaccinia-virus gene in this recombination field virus — it is mostly inserted in the center For this reason, normal hemagglutinin protein is not produced. Moreover, since the inserted non-A-non-B-hepatitis virogene has a start codon ATG in the

fraction of the head, mRNA is newly compounded from here and protein synthesis is performed.
[0022] Screening of the recombination vaccinia virus acquires the recombination virus candidate stock of HA (-) first by hemadsorption examination (HA examination) (Shida, H., virology 150:451-462, and 1986), and can be carried out by the technique of detecting an electropositive clone by the plaque hybridization which uses as a probe the nucleotide sequence which carries out the code of the peptide which subsequently constitutes non-A-non-B-hepatitis virus-antigen protein.
[0023] Rearrange, the vaccinia virus is infected with an animal cell, this invention makes the gene containing the base sequence which was produced still as mentioned above and which cultivates this cell and carries out the code of the non-A-non-B-hepatitis virus-antigen protein discover, and the manufacture technique of the manifestation non-A-non-B-hepatitis virus-antigen protein which consists of collecting manifestation protein is offered.
[0024] The mammalian cell is suitable and, as for an animal cell, a lagomorph kidney passage cell (RK-13 cell) may be used especially suitably. Moreover, a culture condition is determined depending on the animal cell to use, and the culture medium which can be increased, incubation temperature, incubation time, etc. are chosen suitably. For example, in the aforementioned P7.5-E12/RLV, the cow blood serum inclusion EMEM culture medium which contains a fructose instead of a glucose may be used.
[0025] an antibody [as opposed to this antigen protein in a manifestation of non-A-non-B-hepatitis virus-antigen protein] — or it may be checked by the immunological technique of the common use like the immunoprecipitation method using the non-A-non-B-hepatitis patient blood serum containing it, and an indirect fluorescent antibody technique (the drawing 2 and drawing 3)
[0026] this invention offers the manifestation non-A-non-B-hepatitis virus-antigen protein which has especially sugar among the non-A-non-B-hepatitis virus-antigen protein again discovered by doing in this way.
[0027] The manifestation of glycoprotein made this gene expression possible for the first time by invention of the manifestation system which uses the recombination vaccinia virus containing the gene containing the base sequence which is not yet realized in a non-A-non-B-hepatitis virus relation field, and carries out the code of this antigen glycoprotein.
[0028] The manifestation non-A-non-B-hepatitis virus-antigen protein which has "sugar" means including both nature type non-A-non-B-hepatitis virus-antigen glycoprotein and its analog among this specification. moreover, the thing for which the analog of this nature type antigen glycoprotein prevents the irruption to th animal inside of the body of a non-A-non-B-hepatitis virus — or the alteration field of the domain which can produce the antibody to the non-A-non-B-hepatitis virus which can eradicate this virus infected with the animal is meant
[0029] this invention offers the vaccine for treating or preventing non-A non-B hepatitis containing the manifestation non-A-non-B-hepatitis virus-antigen protein which has the further above-mentioned sugar.
[0030] Again, the attenuation of this invention was carried out, or it offers the vaccine for treating or preventing the non-A non-B hepatitis which carried out the inactivation and which rearranges and contains the vaccinia virus.
[0031] The vaccinia virus is a kind of the poxvirus group used to build the Homo sapiens's variola immunity. After proliferating the recombination vaccinia virus in the cell of an animal, an organization, the organum, etc., it can consider as an attenuated metaplasia vaccine, or virulence can be weakened, an inactivation can be carried out with formaldehyde, and it can consider as a killed vaccine. It is actually proved by the below-mentioned example 7 by inoculating recombination vaccinia-virus P7.5-E12/RLV in a lagomorph r gions-of-back hide that the antibody to non-A-non-B-hepatitis virus-antigen protein is produced in a lagomorph blood serum (drawing 4).
[0032] the gestalt of the adjuvant vaccine, like generally, an immunogen is contained in a water in oil emulsion, the vaccine of this invention is adsorbed on inorganic gels, such as an aluminum hydroxide and an aluminium phosphate, or the organic adjuvant of common use is included — or although used with the solution gestalt in liquid solvents, such as a physiological saline and a glycerol, it is not limited to this Moreover, as an application of a vaccine, inoculation is desirable, and a medicine is prescribed for the patient in the amount from which the prevention effect or curative effect made into the purpose is acquired, and it depends for the amount on the age of an individual, weight, a symptom, etc.

[Translation done.]

EXAMPLE

[Example] Although this invention is explained still in detail, this invention is not limited to these examples by the following examples, unless the summary of this invention is changed.
[0034] Example The cloning vaccinia-virus WR stock of the hemagglutinin (HA) gene of one vaccinia virus is refined by the cane-sugar density gradient centrifugation [Joklik.W.K, Virology, 18, and 9-18 (1962)]. a 50mM Tris-HCl (pH 7.4) buffer (it EDTAs 1mM —) It suspends during 0.5% inclusion [sodium-dodecyl-sulfate], and is pro tee ****** K. After incubating at 37 degrees C in addition overnight so that it may become 250-1000microg [/ml] concentration, TE buffer extracted 3 times with bottom phenol [of a saturation]:chloroform (1:1) liquid, ethanol precipitation was performed, and the virus DNA was obtained. It is HindIII in the Nakashio concentration buffer solution about this DNA. It digested and Hind IIIA fragment of about 50 kbs was obtained by the agarose gel electrophoresis. This Hind IIIA fragment was digested by SalI in the high salt concentration buffer solution, and the Hind III-SalI fragment of about 1.8 kbp located in 3' terminal of Hind IIIA fragment was isolated by the agarose gel electrophoresis.
[0035] On the other hand, it is HindIII in the Nakashio concentration buffer solution about a plasmid pUC13. It continued, and in the high salt concentration buffer solution, it digested by SalI and line-ized. this line — the-izing plasmid was isolated by the agarose gel electrophoresis
[0036] the aforementioned Hind III-SalI fragment and a line — a-izing plasmid — the inside of the ligation buffer solution — T4 ligase — connecting — this reaction mixture — using — Escherichia coli JM103 The transformation was carried out. Plasmids were collected from each transformant with the alkali extraction method. Furthermore analysis by the restriction enzyme was performed, the plasmid which has the gene configuration made into the purpose was chosen, and this was named pHA13.
[0037] Example The vector for recombination for incorporating the foreign gene connected with 7.5kD protein promoting agent and its lower stream of a river of the production vaccinia virus of the multi-vector for recombination using 7.5kD protein promoting agent of two vaccinia virus in the genome HA gene of the vaccinia virus was produced by the following technique.
[0038] First, from the vaccinia-virus WR stock, it extracted like the technique which showed DNA in the example 1, and 7.5kD protein promoting agent was isolated by Venkatesan's et al. technique [Venkatesan & B.Moss, J.Virol.33, and 738-745 (1981)]. That is, DNA of extracted WR stock was digested in SalI, and the cloning of the DNA fragment of obtained 0.9kbps was carried out to SalI site of pUC18. Furthermore, this plasmid was digested by RsaI and HincII, and the RsaI-HincII fragment of 0.26 kbp including 7.5kD protein promoting agent was obtained. The cloning of this DNA fragment was carried out to pUC18 (p 7.5-18).
[0039] Next, 7.5kD protein promoting agent who isolated by the above technique was inserted in NruI site of HA gene which carried out the cloning in the example 1 by the following technique.
[0040] They are EcoRI and HindIII in the Nakashio concentration buffer solution about plasmid p 7.5-18. By digesting, it is about 0.29 kbp. 7.5k promoter gene was started and it isolated by the agarose gel electrophoresis. The isolated gene fragment was made to react to the bottom of dTTP, dCTP, dATP, and dGTP presence with Klenow fragment in the nick-translation buffer solution, and the terminal was made smooth.
[0041] On the other hand, plasmid pHA-13 were digested and line-ized in NruI buffer solution. The gene fragment which this line-ized plasmid and the above smoothed is connected by T4 ligase in the ligation buffer solution, and it is Escherichia coli JM109. The transformation was carried out. 7.5kD promoting agent (P7.5) was inserted in the orientation and the same direction of HA gene as a result of analysis according the plasmid obtained from the transformant] to a restriction enzyme. This was named pVR-1 (drawing 1).
[0042] Example The recombination vector used in order to incorporate the gene of the envelope field of the production non-A-non-B-hepatitis virus of 3 recombination plasmid into HA gene of the genome of the vaccinia virus was produced by the following technique.
[0043] Clone C10-E12 (fine ********** of No. 3444) (Japanese Patent Application No. 413844 [two to]) with the field which carries out the code of the structural protein of a non-A-non-B-hepatitis virus was digested in KpnI in the low-salt concentration buffer solution, the gene fragment of about 1.0 kbps was isolated by the agarose gel electrophoresis, and the gene fragment was obtained (drawing 1).
[0044] moreover, the object for recombination — similarly plasmid pVR-1 was digested and line-ized in KpnI in the low-salt concentration buffer solution Both were connected by T4 ligase in the ligation buffer solution. This link object is us d and it is Escherichia coli HB [101]. The transformation was carried out and plasmids were collected in the alkaline process from the transformant. Analysis by the restriction enzyme was performed, the plasmid by which the envelope field is connected with the promoting agent's lower stream of a river in this orientation was obtained, and this was named P7.5-E12.

[0045] Example Plasmid P7.5-E12 of 150microg was extracted from the Escherichia coli cultivated by 200ml culture medium using the manufacture DIAGEN plasmid kit (product made from DIAGEN) of DNA used for the production a transfection of 4 recombination vaccinia virus. This plasmid was refined by CsCl density gradient centrifugation. That is, they are 10 degrees C and 55,000rpm about that with which melted the plasmid in rho= 1.47g [/ml] CsCl liquid (EtBr inclusion), and the quick seal tube (made in Beckmann, an ultra clearance, 13x51mm) was filled up. The at-long-intervals core was carried out at 15:00. (VTi 65.2 rotor, Beckmann ultracentrifuge) . After centrifugal and closed circular The bands of plasmid DNA were collected and deed EtBr was removed for the isopropanol extraction 3 times. Then, the refining plasmid was obtain d by ethanol precipitation.
[0046] It is HindIII about P7.5-E12 which the above refined in the Nakashio concentration buffer solution. It clove. 25microg readiness of this line-ized recombination vector was carried out for transfections.
[0047] b) The introduction of a transfection gene was performed according to Perkes's et al. electroporation method [Marion E.Perkus, Keith Limbach and Enzo Paoletti, J.Virol.63, 3829-3836 (1989)]. Namely, 175cm2 A vaccinia-virus Lister stock is infected with lagomorph ren origin cell-strain RK13 cell which carried out the monolayer culture to the culture bottle by m.o.i.5, and they are 37 degrees C and 5%CO2. After making it adsorb in the bottom for 1 hour, infected cells were collected using the trypsin. HeBS buffer (pH7.05) washed the collected cell twice, and it suspended in 0.8ml HeBS buffer with DNA25microg prepared to transfections by a. The cell suspension was moved to the pulsar cuvette (Bio-Rad make), and it cooled for 10 minutes in this status in Hikami. Then, the pulse of 200V (Capacitance and 960 micro F) was applied once in the ********** pulsar. It cools for 10 minutes again in Hikami, and is 20ml 10% FCS-MEM about a cell. It suspends and is 2 175cm. They are 37 degrees C and 5%CO2 with a culture bottle. It cultivated under presence. The freeze thawing of this culture was repeated 3 times 24 hours after, and viruses were collected.
[0048] It rearranged from the collected virus and the virus was chosen by hemadsorption examination (HA examination). The experimental technique is as follows. The virus was inoculated so that it might become 600 plaques / laboratory dish into RK13 cell which carried out the monolayer culture to 9cm laboratory dish, and it cultivated for two days, and ****** was made to form. Except for the culture supernatant, 0.5% of fowl erythrocyte liquid was added. After incubating for 10 minutes at 37 degrees C, the plaque was observ d and the plaque (recombination virus candidate stock) which does not adsorb an erythrocyte was obtained.
[0049] Next, the recombination [which was obtained previously] virus in which it rearranges, the plaque hybridization of 12 clone is performed among virus candidate stock 34 clones, and the gene was further included from the candidate stock was chosen, having used the gene of a non-A-non-B-hepatitis virus structural-protein field as the probe. First, it rearranged into RK-13 cell which carried out the monolayer culture to 3cm laboratory dish, the virus candidate stock was inoculated so that it might become 20 - 50 plaque / laboratory dish, and it was cultivated for two days, and the plaque was made to form. the formed plaque top — a nylon membrane (high bond N, product made from Amersham) — carrying — a plaque — a membrane top — moving — 0.5N 1M Tris-HCl (pH7.4) after having processed for 5 minutes by NaOH and making DNA denaturalize — neutralizing — further — 1.5M NaCl and 0.5M Tris-HCl (pH 7.4) It processed and DNA was made to stick to a membrane. 305 nm after air-drying a membrane Ultraviolet rays were irradiated for 5 minutes and DNA was fixed to the membrane. This membrane was dipped in the hybridization buffer and it incubated at 65 degrees C for 1 hour. Furthermore, moved the membrane to the hybridization buffer which added the gene of the non-A-non-B-hepatitis virus structural-protein field which carried out the indicator by 32P using the random prime labeling method, and it was made to react at 65 degrees C overnight, and rearranged with probe DNA, and the virus DNA was made to hybridize. it washed twice every / during 10 minutes] at 1xSSC, 0.1% SDS, and 65 degrees C, autoradiography was performed at -70 degrees C, and the electropositive clone was detected
[0050] Consequently, five clones are positivities and it is P7.5-E12/RLV about a clone, respectively. It was named #7, and 8, 9, 10 and 11.
[0051] They are 0.05% trypsin and 0.1mM about RK-13 cell in which the manifestation by example 5 indirect fluorescent antibody technique carried out the authentication monolayer culture. After processing with EDTA solution and making it a single cell, it distributes so that it may become [ml] MEM culture medium (5% calf serum, 0.22% sodium hydrogencarbonate) in 50,000 pieces /. They are the Lister stock of a vaccinia parent strain, and P7.5-E12/RLV to this cell solution m.o.i.=0.1 It inoculates separately so that it may become. The cell into which the virus was inoculated is put on 12 hole slide glass in 20microl / hole, and they are 37 degrees C and 5%CO2. One evening is cultivated in the bottom. Distilled water washes the cultivated slide glass once, and after air-drying, it soaks in a methanol mixture for 15 minutes -20-degree C 5% acetone and 50%, and fixes. Are air-dry after fixation, and the non-A-non-B-hepatitis patient blood serum diluted with PBS (-) 50 times is carried 20microl / hole every, and is made to react for 40 minutes at 37 degrees C. It washes 3 times by PBS after a reaction for 40 minutes (-), and anti-Homo-sapiens IgG and FITC indicator (goat) diluted with PBS (-) 250 times are carried 20microl / hole every, and is made to react for 30 minutes at 37 more degrees C. Although fluorescence was not accepted in the cell with which the Lister stock was infected when it washed 3 times by PBS after a reaction end (-) and having been observed with the fluorescence microscope, unique fluorescence strong against the cell with which P7.5-E12/RLV was infected accepted (drawing 2).
[0052] One evening of authentication RK-13 cells of the manifestation by the example 6 immunoprecipitation method was cultivated by the cow blood serum EMEM culture medium 5%, and the mono-layer was made to form. It is 100microcurie to EMEM which the Lister stock or P7.5-E12/RLV is infected with this, and

contains 10mM fructose instead of a glucose. The 3H-glucosamine was added and it cultivated for 16 hours. Unique antigen protein was made to sediment by the immunoprecipitation method using a normal people blood serum or a non-A-non-B-hepatitis patient blood serum, and protein A sepharose, and it analyzed by the SDS-polyacrylamide-gel-electrophoresis method (SDS-PAGE). It was checked that the specific band of gp35 is accepted only in what, as a result, carried out the immunoprecipitation of P7.5-E12 / the RLV infected cell by the non-A-non-B-hepatitis patient blood serum, and it is discovered (drawing 3).
[0053] 108 PFU was inoculated for immunoprecipitation examination P7.5-E12/RLV using example 7P7.5-E12 / RLV immunity lagomorph blood serum in th_ regions-of-back hide of the Japanese white kind lagomorph (1.5-2.0kg), and the blood serum was extracted two months after.
[0054] On the other hand, it is on T7 promoting-agent lower stream of a river of a blue script KS vector (Strategene). C10-E12 DNA It inserts and is an in vitro transcription. C10-E12DNA Complementary RNA was compounded. It is with this compound RNA and the in vitro translation is performed in a lagomorph ********** site extract. The synthesis in a test tube of the polypeptide by which a code is carried out by C10-E12 DNA was performed.
[0055] The immunoprecipitation examination was performed like the example 6 using the polypeptide compounded with the lagomorph immune serum obtained previously (drawing 4).
[0056] as shown in the lane 2 as a result, into the blood serum of P7.5-E12 / RLV immunity lagomorph, the code of the structural-protein field of a non-A-non-B-hepatitis virus is carried out C10-E12 DNA **** — it has checked that the antibody which reacts specifically with the built polypropylene theine was produced

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[Translation done.]